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Autonomous Pathogen Detection System (APDS)

FY[redacted] Annual Progress Report

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Budget: FY[redacted] - $4,000,000
FY[redacted] - $4,300,000 (anticipated)

[redacted]

OBJECTIVE

The Autonomous Pathogen Detection System (APDS) is targeted for domestic applications in which the public is at high risk to exposure from terrorist releases of bioagent (such as mass transit, office complexes, etc.), and as part of a monitoring network for urban areas and major gatherings (inaugurations, Olympics, etc.). This system will be a stand-alone unit capable of continuously monitoring airborne biological threat agents. The APDS will be completely automated, offering continuous or on-demand aerosol sampling, automated fluidic sample handling and transport, multiplex flow cytometry immunoassay, multiplex nucleic acid recognition (PCR), and automated data processing and reporting.

The APDS project consists of two parallel development paths. The first is a staged effort to design, construct, and test several evolutionary versions of the APDS with increasing detection and identification capability. Three distinct versions of the APDS will be constructed and tested as key components mature and become integrated into the APDS architecture. APDS-I (single-plex immunoassay using flow cytometry) was built and field tested in [redacted]. The second version of the APDS will be completed by the end of [redacted], and will be capable of performing *automated multiplex* immunoassays (end [redacted]) and *flow-through PCR* (end [redacted]). This version (APDS-II) will be demonstrated at a major public event in [redacted]. The APDS-III corresponds to the final APDS prototype and will incorporate a *multiplex PCR* capability and fluidics that support commercial viability of the technology. This version is slated for completion at the end of [redacted] In [redacted], the APDS-III will be largely dedicated to testing and commercialization .

The second development path is to initiate basic R&D that will spawn the next-generation technologies necessary to enable a miniaturized version of the APDS. Current efforts are focussed on developing sample preparation methods that will perform mixing and collection for the bead-based flow cytometry immunoassay format and DNA concentration and purification for PCR analysis in a miniaturized (MEMS – micro electro-mechanical systems) format. Novel acoustic methods are used to provide separation and mixing functions, dielectrophoretic (DEP) particle capture is used to concentrate and purify the samples, and magneto-hydrodynamic (MHD) pumps are being investigated as a means of pumping and switching the fluid through the flow channels in the system. These devices feature no moving parts that might clog or fail. The sample preparation module will be completed in [redacted]. At that point, a decision will be made to either insert the sample preparation module into the baseline APDS architecture (for demonstration) or adapt it to an equally miniaturized detection system.

PROGRESS REPORT

We have adopted a modular approach in our development of the APDS-II. Separation of the modules is based on their functionality (i.e., aerosol collector, sample preparation fluidics, nucleic acid assay, and multiplex flow cytometry immunoassay). This approach allows us the flexibility to develop and optimize each module before it is integrated into the final autonomous system. In general, most of this development work will occur in the first three quarters of [redacted], with integration to occur in the fourth quarter. In-line nucleic acid recognition (PCR) will not be integrated into APDS-II until [redacted] The primary objective for [redacted] is to develop and demonstrate an integrated instrument containing an aerosol collector, sample preparation module (fluidics), and multiplex immunoassay. In this approach, the aerosol collected sample is added to a collection of microbeads. Each color of microbead contains a capture assay that is specific for a given bioagent. Fluorescent labels are then added to identify the presence of each agent on

the bound bead. Each optically encoded and fluorescently labeled microbead is then individually read in the flow cytometer.

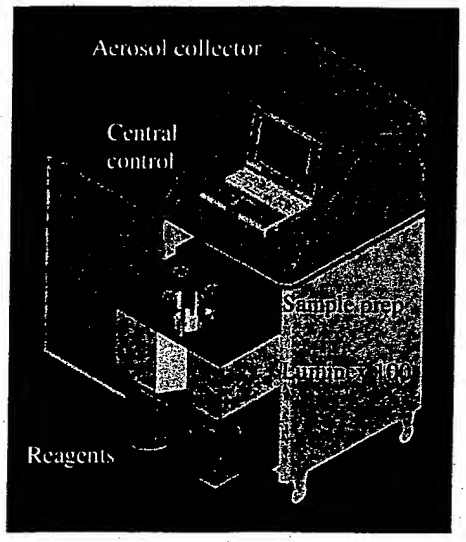

Figure 1: Integrated APDS system

We have completed a preliminary design for packaging and physically connecting the different components of the APDS-II system in a simple, "ATM" style chassis (Figure 1). Concerns such as heat transfer, vibration isolation, fluidic connections, flexibility, and user access are addressed in this design. Space for future modules, such as the in-line nucleic acid assay, has also been allocated in this chassis. The final design review will be conducted in mid-July, with procurement and assembly completed by early- to mid-August.

One of the most challenging tasks of the entire APDS development project is the unification of the separate components under a central control system. We chose National Instrument's Labview software as the common programming language for APDS, and have currently written software to control both the aerosol collector and sample preparation module. The largest component, reprogramming the LX100 multiplex flow cytometer in Labview, is currently in progress. The graphical user interface (GUI) is complete and currently undergoing a second round of improvements. We are able to read, display, and control several functions of the LX100 instrument. We have also defined the minimum set of control and monitoring functions necessary to conduct an autonomous multiplex assay, and expect to complete our "alpha" version of the LX100 Labview control software by late July.

APDS-II Modules

Aersosol collector module

The aerosol collector used in APDS-I contains an LLNL designed virtual impactor for selectively sampling a given particle size range coupled to a SASS-2000 (Smart Air Sampler System, Research International) aerosol collector. The first development task of [redacted] was to compare the performance of this LLNL/SASS Hybrid against two other collectors, the SASS-2000 alone, and the SCAEP (Space Charged Atomizing Electrostatic Precipitation, Team Technologies) model PM-1B. The industrial standard All Glass Impinger; AGI-30 (Andersen Laboratories) was used as the reference sampler. Side-by-side system performance comparisons conducted in two different field trials at the Harry Reid Center for Environmental Studies, University of Nevada-Las Vegas (UNLV).

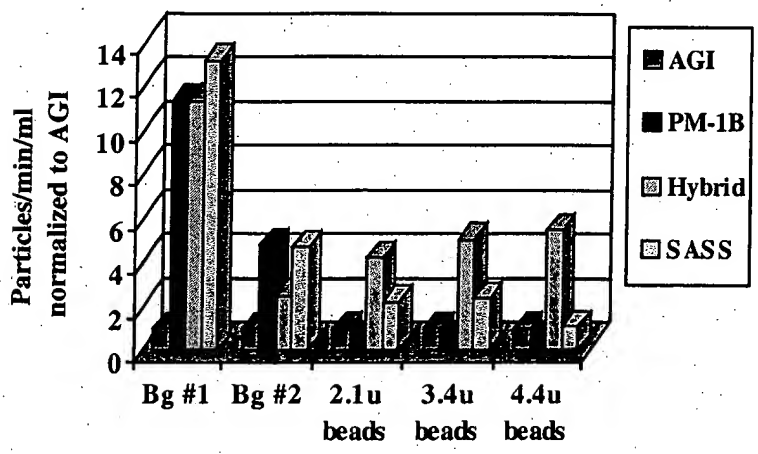

Figure 2: Side by side comparison of aerosol collectors from UNLV field trial

The results indicated the LLNL /SASS Hybrid has the best overall collection rate for the different samples and particle sizes tested (Figure 2). Custom modifications to the LLNL/SASS Hybrid instrument for optimum adaptation to the APDS-II are nearing completion. Some of the more important upgrades to the LLNL/SASS Hybrid, include, increased air flow, variable particle size selection, smaller packaging, onboard particle counter, ruggedized design, and autonomous Labview-based control system (Table 1). Once the aerosol collector has been assembled and initial function checks have been completed (late July) we have planned a second round of field testing at the UNLV test facility. We will simultaneously release a wide distribution (1-10 μm) of polystyrene bead sets into the aerosol distribution chamber. The collected samples will be analyzed using multiplex flow cytometry to determine the collection efficiency as a function of particle size. One major advantage of the APDS-II aerosol collector is the ability to change the bypass-to-product ratio of the input air, providing finer control over the particle size selection. The collector will be integrated with the remaining components of the APDS-II system by mid-August.

Table 1: Technical specifications for the APDS-II aerosol collector

APDS-II aerosol collector
cyclone-type collector
124 CFM (3513 lpm) Total air flow.
117 CFM (3313 lpm) bypass fan.
Computer controlled adjustable bypass/ product ratio from 0:1 to 10:1 (This covers the entire design range of the LLNL pre-fractionator).
0.87 ft.^{3} physical volume at <21 lbs.
Built-in hot wire anemometer flow measurement of bypass and product flows
Internal CPU. Network ready.
Sealed flow only contaminates components that can be sterilized. Better seals.
Ruggedized construction, Mil-Spec hardware was specified.
Meets and exceeds performance, plus corrects problems of earlier Hybrid.

Sample preparation (fluidics) module

The multiplex immunoassay protocol will be used to determine the sequence of actions performed by the sample preparation module. Since engineering of the sample preparation module and assay development are necessarily parallel efforts, we defined a generalized set of operations for the sample preparation module that is flexible enough to cover virtually any defined protocol. These operations include:

- extracting a portion of the collected sample volume from the aerosol collector
- capturing the sample with antibody-labeled microbeads
- labeling the samples with fluorescent reporter antibodies
- separating, washing, and pre-concentrating microbeads to improve assay performance and prevent cross-contamination between measurements
- flowing the resulting mixture to the flow cytometer for analysis

Early in the project, we identified that the approach used in APDS-I was neither flexible nor modular enough to accommodate the added complexity of operations required for APDS-II. Based on experience with our previously developed in-line PCR system and some early bench

testing, we adopted a technique advocated by a collaborator (Global FIA, Gig Harbor, WA) using a sequential injection analysis (SIA) platform. The basic components of this platform include: a carrier fluid, a syringe pump, a holding and mixing coil, a multi-port selection valve, reagent/sample storage, and separation cell (see Figure 3). The carrier fluid is used to draw and pump fluids sequentially through the various sample ports on the selection valve. Aliquots of air are used to spatially separate the carrier from reagent and sample volumes, greatly minimizing the chance of cross-contamination. The holding/mixing coil serves to mix various assay components (i.e., sample, microbeads, reporter, etc.), to perform incubation (both heated and unheated coils are being tested), and to prevent contamination of the syringe pump. The selection valve serves as the interface between all components of the sample preparation unit, offering a flexible medium for changing and upgrading the various fluidic components. The separation cell is used to separate, wash, and/or preconcentrate the microbeads at various points in the multiplex immunoassay.

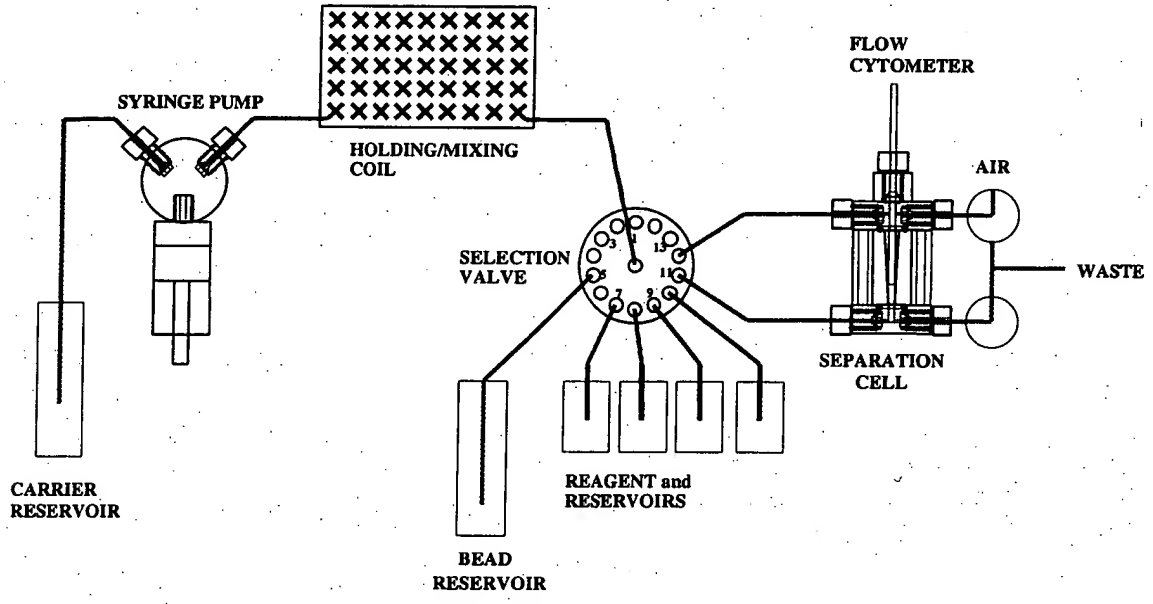

Figure 3: SIA based sample preparation module for APDS-II

Using a simple serial based Labview control system and modified Global SIA hardware, we constructed a fluidics testbed to resolve such critical issues as bead separation, cross-contamination, mixing and incubation. The most challenging issue was the ability to sequester or wash the microbeads at various steps in the assay process without diluting the sample (resulting in decreased sensitivity), losing the microbeads (resulting in cross-contamination, decreased sensitivity, and clogging of the filters), or introducing undue backpressure on the fluidics lines. We have evaluated a number of different separation techniques, including conventional matrix type filters, flat filters, MEMS-based separators, and magnetic separation columns (Table 2). Metrics for these tests include bead collection efficiency, bead recovery from the filter, and backpressure.

The two most promising candidates for bead manipulation (Table 2), the MEMS separator and the magnetic separation column, are both novel concepts developed internally at LLNL. The MEMS separator physically retains the microbeads by using narrow, lithographically formed structures to pass particles less than less than 3 μm in diameter. The results obtained using these MEMS devices were excellent, with less than 1% bead loss and greater than 90% bead recovery.

The magnetic columns also functioned well as bead separators, yielding both high collection efficiencies and bead recovery values. Lab bench tests also demonstrated that the magnetized microbeads did not interfere with assay performance. Given the encouraging results for both the MEMS and magnetic separation technologies, we are currently extending our experimental protocols to evaluate such long-term performance metrics as repeatability, clogging and leakage.

Table 2: Experimental data comparing various in-line bead separation technologies

Separation Technology	*Collection Efficiency (%)*	*Bead Recovery (% retentate)*	*Backpressure*
Matrix filter (stainless steel)	95-50	6-28	Low-moderate
Matrix filter (ultra high molecular weight polyethylene - UHMWPE)	100-90	30-52	Moderate
Matrix filter (polyetheretherketone - PEEK)	83-82	50-51	Moderate
Matrix filter (cellulose)	100	45-65	Moderate
Flat filter (nucleopore)	100	10-12	Severe
MEMS separator	100-99	91-94	Low-moderate
Magnetic filter	98-97	79-80	Moderate

Our initial experimental data indicates that cross-contamination is easily avoided by rinsing the exposed fluidics line with a minimal volume of carrier fluid between operations. We will complete experimental evaluation of the sample preparation module by late July, with full integration into the APDS-II system by early to mid-August.

Multiplex flow cytometer module and immunoassay development

A major advancement related to large-scale multiplex immuno-analysis is the recent release of the Luminex, model LX-100 flow cytometer. This instrument, which was not available when we developed the APDS-I, has a 100-plex target capability. The LX-100 (Fig. 4) constitutes the centerpiece of our development effort for incorporating multiplex flow cytometry into the APDS-II system. Luminex provided a complete optical and fluidic upgrade for the LX-100 flow

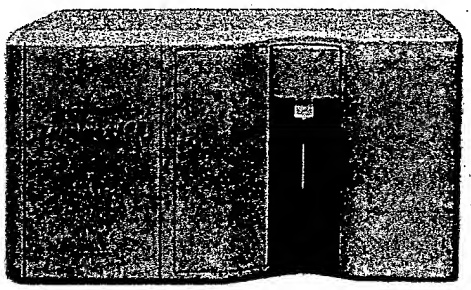

Figure 4: LX-100 flow cytometer

cytometer in [redacted]. Luminex also extended their bead sets from 25 to 100 sets in May of 2000. We have recently obtained samples of all 100 bead sets, and a mixture of all bead sets has been

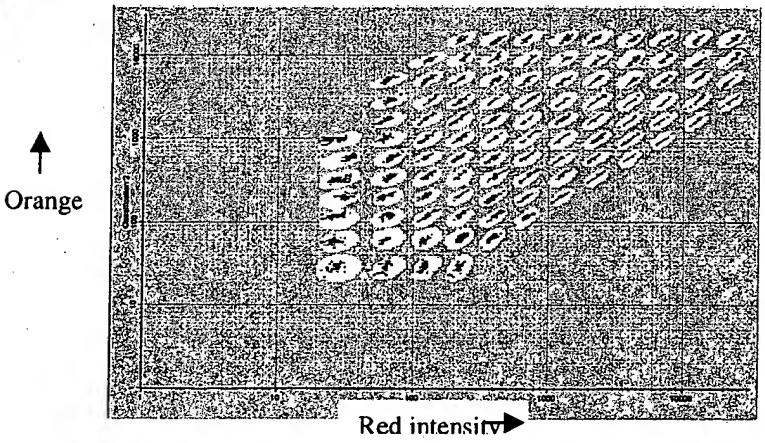

Figure 5: Dot plot of a mixture of 100 bead sets depicting adequate separation of each bead set

successfully analyzed at LLNL (Fig. 5).

The current focus of our assay development for [redacted] is the demonstration of an automated 7-plex detection (4 bioagent simulants, 3 controls) capability. The result shown in Figure 6 is close to achieving that goal. Bead sets were coated with specific antibodies for *Bacillus globigii* (Bg), *Erwinia herbicola* (Eh), bacteriophage (MS2), Ovalbumin (Ov), and selected fluorescent reporter dyes. We also coated two separate bead sets with reagents that provide internal positive and negative controls for each step in the sample preparation process. These controls will provide a continuous monitor of the APDS-II operation even if no target agents are detected. Although these results are extremely encouraging, many challenges remain in the areas of assay development and extended multiplexing. The final assay protocol for FY00 will be completed by early- to mid-August, and its performance characteristics evaluated in a blind laboratory trial in [redacted]

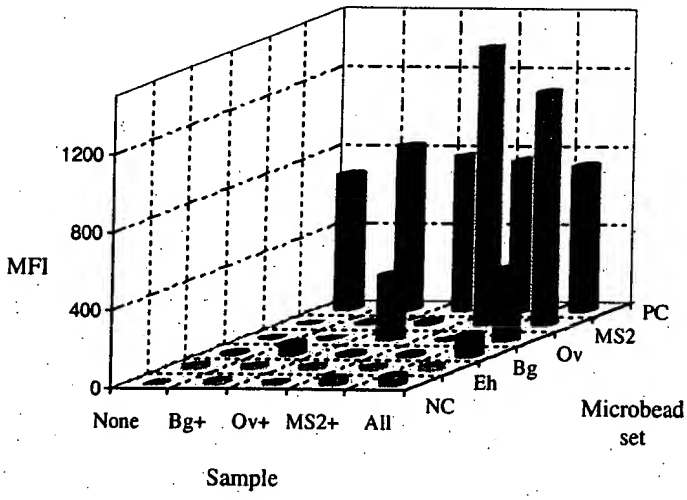

Figure 6: Luminex LX-100 analysis of 4 simulants using a 6-plex assay. Median fluorescent intensities (z-axis) of each microsphere set (y-axis) analyzed from 5 different samples (x-axis)

Flow-Through PCR Development

Flow-through PCR provides a method for conducting continuous, real-time nucleic acid assays. This technology will add an orthogonal detection technique to the multiplex immunoassay capability, thereby increasing the reliability of a positive identification and providing additional information regarding the origin and characteristics of a detected pathogen. Flow-through PCR will be integrated into APDS-II in [redacted]. This year's effort [redacted] has been primarily directed at design, construction, and characterization of the first generation flow-through PCR system.

The flow-through PCR system consists of a LLNL-designed, silicon-machined thermocycler mounted in-line with a sequential injection analysis system (Figure 7). The SIA system performs all necessary sample preparation functions (mixing of sample with PCR reagent components, etc.) and delivers the prepared PCR reagent/sample aliquot to the thermocyler unit. The thermocycler is designed with appropriate light sources and detectors to perform real-time TaqMan assays. After

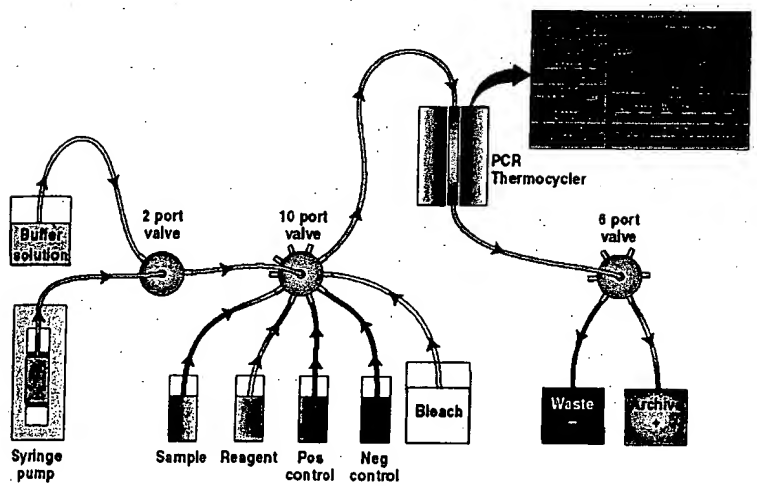

Figure 7: Flow-through PCR system

completion of the assay, the SIA system decontaminates the thermocycler chamber and all exposed fluid delivery tubes.

The flow-through PCR system has undergone extensive characterization during FY00. We successfully demonstrated amplification in 10^6 cfu/ml of several different bacterial species, including Bacillus globigii (*B.g.*), Bacillus thuringensis (*B.t.*), and Erwinia herbicola (*E.h.*). Amplification with as little as 10 μl of sample (7μl reagent, 3μl sample) has been successfully performed. We have also determined that the chamber and sample port can be quickly decontaminated by flushing with small volumes of bleach, making possible repeated cycling of positive and negative controls through the same sample port (Figure 8). This operation has recently been automated so that only a positive assay result (defined by a threshold fluorescent amplitude value) triggers a decontamination cycle. Ultimately, the flow-through PCR system will be autonomous, requiring less sample preparation by the user. Toward this end, we have demonstrated that in-line mixing of the reagents and the sample give comparable signals to premixed reagent/sample reaction volumes.

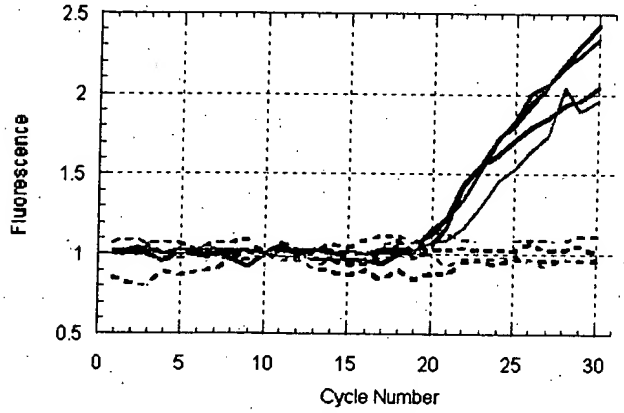

Figure 8: Taq-Man PCR assay of alternate negative controls (dotted lines) and positive controls (solid lines)

We are currently in the process of developing the next generation flow-through PCR system. This system will be based on the latest miniaturized PCR technology developed at LLNL, the Handheld Advanced Nucleic Acid Analyzer (HANAA) platform. The HANAA has dual, independently controlled thermocycle chambers, multiplex dual wavelength detection, and a faster, more thermally efficient thermocycler chamber design. Design modifications necessary for converting the HANAA to a flow-through PCR format have been completed. Procurement and fabrication of the new flow-through system will be complete by the end of this fiscal year.

Technology Development of a Miniaturized Fluidics Module

Several key enabling technologies must be developed if we are to ever realize a miniatured version of the APDS. As a starting point, we have selected the development of a MEMS-based Fluidic Module (MFM) to provide the sample preparation functions for the APDS immunoassay and PCR detection in a compact format. The technologies chosen to perform the sample preparation functions of mixing, concentration, purification, pumping and valving are unique to MEMS devices. They are enabled by the dimensions associated by MEMS (20-200μm) and perform these functions with no moving parts to wear or to obstruct flow. A layout of the MFM system is shown in Figure 9. Sample enters the system from the aerosol collector, passes through an acoustic fractionator to separate the target particles, and proceeds to an immunoassay assay or a PCR assay for identification. In the immunoassay leg, the particles are acoustically mixed with antibody-coated beads, then held using dielectrophoretic (DEP) concentration for washing and addition of reporter antibodies. In the second leg, the particles are held using DEP while lysing solution is introduced to break open the spores and bacteria to release the DNA. The resulting DNA is then trapped by the DEP during a wash and purification step.

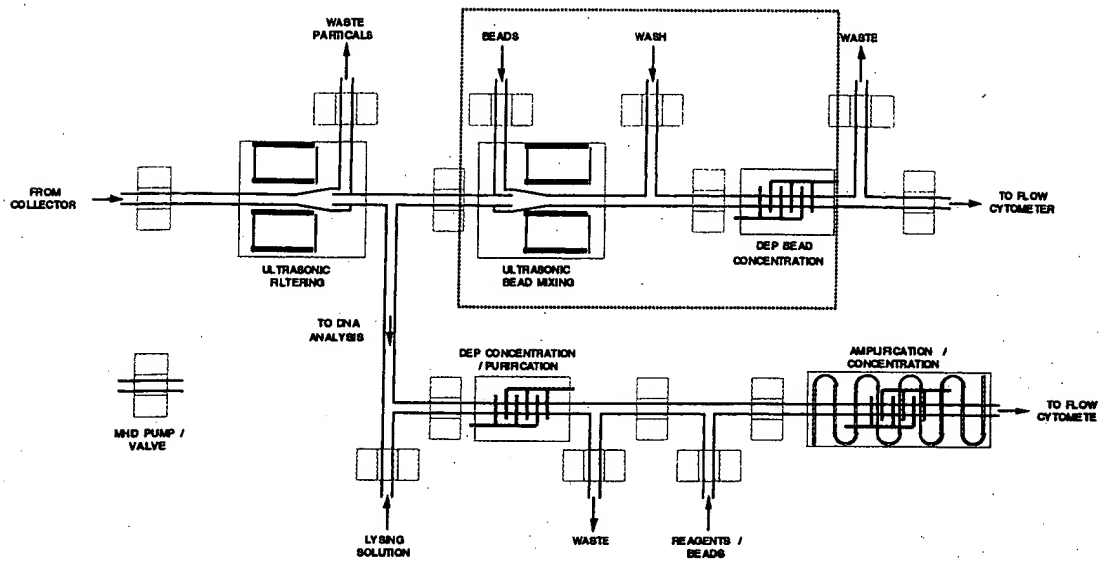

Figure 9: MFM system diagram. The integrated prototype for FY00 is shown in the dotted box.

During this first year, the research emphasis has been in performing proof of concept experiments on the various technologies and resolving manufacturing and materials issues. Preliminary system integration issues are being evaluated through construction of an integrated device that provides the functions of the immunoassay leg of the device. Below we summarize progress in the four major topic areas of DEP concentration, acoustic mixing, magnetohydrodynamic (MHD) pumping, and material fabrication and packaging.

Dielectrophoretic Concentration

We have demonstrated dielectrophoretic concentration of a spore *Bacillus globigii,* a bacteria *Erwinia herbicola*, and DNA molecules on glass substrates. An example of this data is shown in Figure 10, where electrodes capturing *B.g.* spores (10^8 spores/ml) over a 5 minute period is demonstrated.

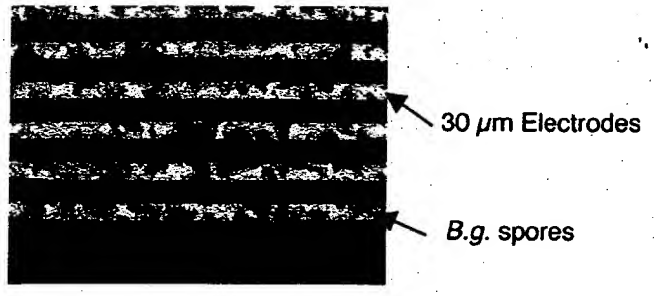

Figure 10: DEP based *B.g.* capture

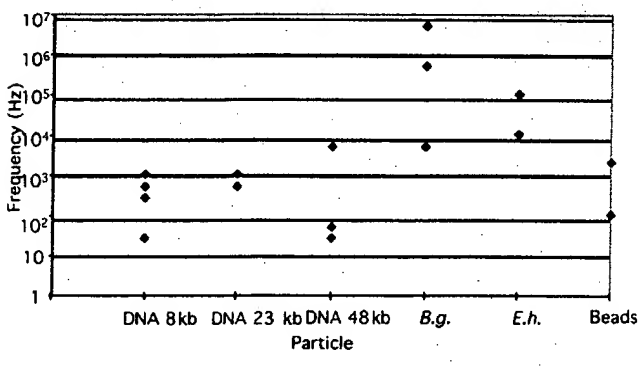

Figure 11: Frequencies at which positive DEP was observed for particles in water.

We have also determined the optimum frequency range for trapping DNA, *B.g.*, *E.h.* and 1 μm polystyrene beads in distilled water using positive dielectrophoretic concentration (Fig. 11). The smaller DNA molecules, having a lower effective dipole, were trapped most efficiently at lower frequencies (0.1 -1 kHz) than the larger *B.g.* spores and *E.h.* bacteria (>1 kHz). Polystyrene beads were captured only at very low frequencies.

Acoustic Mixing

We have demonstrated a non-contact method of manipulating particles in a plastic microfluidic chamber employing acoustic radiation pressure. In [redacted] we developed plastic fabrication processes, designed a removable method of coupling acoustic energy into the sample chamber, and designed and characterized mixing chambers. We achieved bead mixing for a variety of bead sizes (1-10 μm) using resonance frequencies of staggered piezoelectric transducers (Fig. 12). The ability to mix 1 μm sized particles indicates we will be able to mix spore samples with the micron-sized beads used for flow cytometry analysis.

Figure 12: Acoustic mixing of 10 μm polystyrene beads

Magnetohydrodynamic Pumping

For [redacted], the magnetohydrodynamic (MHD) pump milestone was the demonstration of a prototype in the second quarter. Since MHD pumps and switches were recently demonstrated in separate research effort, we focused on the development of high-field magnets and new fabrication techniques to improve MHD performance and integration. Miniature magnets were constructed using metallic glass to reduce losses in the magnetic core during AC operation, and these were tested under simulated operating conditions.

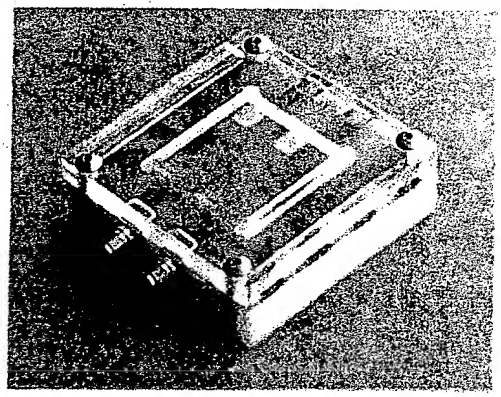

Figure 13: MFM prototype

Manufacture, Assembly, and Packaging

In FY[redacted] we developed a MEMS-based fabrication process that includes channel etching, platinum metallization, and bonding. Acrylic was chosen as the base substrate due to excellent biocompatibility, manufacturability, and current widespread use in this type of application. We constructed a universal package for the testing of individual DEP and MHD devices and integrated DEP/acoustic devices (Fig 13) containing multiple fluidic and electrical ports.

CHANGES FROM THE LIFECYCLE

In general, progress on the individual research tasks and system integration is tracking closely with the projected schedule outlined in our [redacted] lifecycle plan. We expect our major deliverable, the APDS-II with multiplex flow cytometry capability to be completed by the end of [redacted], as originally proposed. However, one notable change regarding the sample preparation module task deserves comment. In the lifecycle plan we proposed to develop a cheap, disposable plastic sample preparation (fluidics) module. Our investigations during [redacted] have convinced us that the sequential injection analysis approach described above for both the immuno and flow-through PCR efforts provides greater flexibility and added functionality for multiplex analysis by APDS-II. Thus, we have stopped work on the disposable module and are concentrating our efforts on the SIA fluidics platform.